Virulence of Bacteria Associated with the Formosan Subterranean Termite (Isoptera: Rhinotermitidae) in New Orleans, LA

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ABSTRACT Examination of Formosan subterranean termites, *Coptotermes formosanus* Shiraki, for possible biological control agents revealed the presence of 15 bacteria and one fungus associated with dead termites from New Orleans, LA, USA. All but one of the bacteria species were gram-negative bacteria. Bacterial isolates from dead termites were primarily *Serratia marcescens* Bizio that caused septicemia in *C. formosanus* and also appeared to contain proteolytic enzymes. Multiple strains of *S. marcescens* were isolated. Six of the eight strains of *S. marcescens* were red, probably not pathogenic in humans, and candidates as biological control agents for *C. formosanus.* Bacteria isolated from termite substrata included *Corynebacterium urealyticum* Pitcher, *Acinetobacter calcoacet/baumannii/Gen2* (Beijerinck), *S. marcescens,* and *Enterobacter gergoviae* Brenner. Some of these bacteria are potential human pathogens. Forced exposure bioassays demonstrated that the T8 strain of *S. marcescens* killed 100% of *C. formosanus* by day 19.

KEY WORDS *Coptotermes formosanus,* termite, bacteria, fungus, *Serratia marcescens,* bio-control

THE FORMOSAN SUBTERRANEAN termite, *Coptotermes formosanus* Shiraki, is the most destructive subterranean termite pest where it occurs (Anonymous 1966). Since its introduction into New Orleans, LA, shortly after World War II, it has become a widespread pest in that area (La Fage 1987). Lack of success in controlling *C. formosanus* using conventional termiticides has led to exploration of alternate control strategies, including baiting, mechanical barriers, and microbial control. Biological control against termites has been unsuccessful (Grace 1997). Reasons for control failures include termite grooming, isolation of infected colony members by walling off diseased termites (Wood and Sands 1978, Su et al. 1982), and difficulty obtaining pathogens because of their cryptic habits. Identification of microbes isolated with *C. formosanus* allows evaluation of their control potential as stand alone pathogens, applied in combination with existing chemistries, or applied in combination with other pathogens. One agent may weaken the termites allowing another opportunistic microbe to become pathogenic (Sands 1969).

Bioassays were conducted to identify the most virulent strains of microbes for biological control of *C. formosanus* and potentially for genetic manipulation and optimization as control agents. Here we report virulence of microbes isolated with *C. formosanus.*

Materials and Methods

Termites. Microbial isolation was conducted with *C. formosanus* obtained from a colony at the Southern Regional Research Center in New Orleans, LA. A bucket trap was established (Su and Scheffrahn 1986) to allow access to termites. One hundred and fifty *C. formosanus* workers of at least third instar (as determined by size) and a slice of spruce (*Picea* sp., 10 by 10 by 2 mm) as food were added to glass petri dishes (100 by 20 mm) half-filled with moistened vermiculite (Hoffman, Landsville, PA). After 30 d, several moribund and dead termites with pink coloration appeared. Bioassays were conducted using six colonies of *C. formosanus* obtained from field sites in New Orleans, LA (i.e., United States Department of Agriculture's Southern Regional Research Center and from the University of New Orleans, New Orleans, LA). *C. formosanus* were collected from bucket traps (Su and Scheffrahn 1986) and maintained on stacked, water soaked, spruce (*Picea* sp.) slats (10 by 4 by 0.5 cm) in plastic containers (13 by 13 by 4 cm) maintained at 26.6°C and ≈100% RH. Termites were identified using keys for soldier identification from Scheffrahn and Su (1994) and Su et al. (1997).

Termite Microbial Isolates. *Isolation Method 1: Slide Streak.* A dead red-tinted worker was placed in a drop of deionized (DI) H_2O on an ethanol washed slide. The termite was transferred to another drop of DI H_2O on an ethanol-washed slide. The termite was cut in half with a sterile surgical blade. The slide was examined at 100× magnification with no cover slip. Bacteria were observed streaming around the termite cadaver. An inoculating loop was placed into the stream and streaked on nutrient agar (Difco 1984) amended with 0.25% glucose (general bacterial growth medium). Isolated bacterial colonies were restreaked on amended nutrient agar and violet red bile agar (VRBA,

Difco 1984), the latter being selective for Enterobacteriacea. Inoculated agars were incubated for 24 h at 24 ± 1°C. Sub-samples were placed in 40% glycerol with a sterile loop, vortexed, and placed in cryostorage (−81°C).

Isolation Method 2: Nutrient Agar. Dead red-tinted workers were plated on amended nutrient agar and water agar (1.8% Moorhead agar, Van Nuys, CA, and DI H_2O). Bacterial growth was transferred to nutrient agar. Isolated colonies were restreaked on nutrient agar and VRBA. Agars were incubated for 24 h at 24 ± 1°C. Isolated sub-samples were stored at −81°C in 40% glycerol solution.

Vermiculite Microbial Isolates. Vermiculite samples from the container in which termites were maintained were plated on amended nutrient agar and water agar (1.8% Morehead agar and DI H_2O). Control vermiculite was plated on both agars. Bacteria could be differentiated from fungi as they appeared shiny or fibrous, respectively. Bacteria were transferred to nutrient agar. Fungi were transferred to acidified potato dextrose agar (amended with six drops 85% lactic acid in 500 ml of APDA, Difco 1984). Isolated subsamples were placed in storage at −81°C as before.

Identification of Bacteria. Bacteria were grown at 30°C for 24 h on nutrient agar. Bacterial isolates were classified as gram-positive or negative with KOH testing (Holt et al. 1994). Bacteria were identified with Biolog Micro Station System (BMSS; Release 3.5, Biolog, Hayward, CA). BMSS is a 96-well microplate evaluating microbial utilization (oxidation) of 95 different carbon sources. MicroLog's database contain libraries of bacterial oxidation profiles. Similarity values are calculated as a value from zero to one. Higher similarity values indicate a more precise identification. Similarity values of >0.5 are considered an acceptable identification with 24-h incubation (Biolog). Cryostored gram-negative isolates were streaked onto Biolog universal growth medium (BUGM, Biolog) + 5% sheep's blood medium. Cryostored gram-positive isolates were streaked onto tryptic soy agar + 5% sheep's blood medium. All plates were incubated at 30°C for 24 h. Positive controls were performed with the following standards: Gram-negative kit from Biolog; *Alcaligenes xylosoxydans* ssp. *xylosoxydans* (Yabuuchi & Yano) ATCC # 27061 and *Providencia stuartii* (Buttiaux), ATCC # 33672. Gram-positive kit from Biolog contained *Rodococcus* sp., ATCC # 6939 and *Jonesia dentrificans* (Prévot), ATCC # 14870.

Bacteria swabbed from agar plates were transferred to 18 ml of 0.85% saline. Inoculum density was adjusted to Biolog turbidity standards. Cell suspensions were inoculated on plates with 150 μl of cell suspension, incubated (30°C for 24 h), and read visually. Growth reactions were analyzed with Biolog.

Termite Bioassays. Virulence of isolates was evaluated against *C. formosanus* in two experiments. Experiment 1 tested 14 isolates. Experiment 2 tested three strains from experiment 1. Cryogenically stored isolates were streaked onto nutrient agar plates (three plates per isolate) and grown for 24 h at 30°C. Inoculum was harvested by collectively washing (10 ml 0.1% peptone solution). Inoculum was used in termite bioassays. The inoculum concentration was determined to be 10^{10} colony formation units per ml (CFU/ml) as determined by serial diultion and plating on nutrient agar. Whatman No. 1 filter paper (82 mm diameter) in a plastic petri plate (100 by 15 mm) was inoculated with 1 ml resulting in 1.89×10^8 CFU/cm^2. Ten *C. formosanus* workers of more than third instar, as determined by size, were added to each treatment. Each dish was replicated three and six times for experiments 1 and 2, respectively. Termites for each replicate originated from a different *C. formosanus* colony within a treatment. Sterile 0.1% peptone was

Table 1. Qualitative results of bacteria isolated by slide streak from *C. formosanus* on nutrient agar seed plates

Plate	Comments	Gram reaction (+ or −)	Closest species ID	Similarity[a]
T1	Red colonies on nutrient agar seed plate streaked from an isolated purple colony on VRBA	−	*Serratia marscescens*	0.852
T2	Red colonies on nutrient agar seed plate streaked from an isolated purple colony on VRBA	−	*Serratia marscescens*	0.905
T3	Red colonies on nutrient agar seed plate streaked from an isolated purple colony on VRBA	−	*Serratia marscescens*	0.752
T4	Red colonies on nutrient agar seed plate streaked from an isolated purple colony on VRBA	−	*Serratia marscescens*	0.832
T5[b]	Cream-colored colonies that turned red: developed slowly on nutrient agar	−	*Serratia marscescens*	0.527
T5A		−	*Serratia marscescens*	0.850
T5B		−	*Klebsiella terrigena*	0.620
T6		−	*Serratia marscescens*	0.562
T7		−	*Serratia marscescens*	0.837
T8		−	*Serratia marscescens*	0.780
T9		−	*Serratia marscescens*	0.573

[a] A similarity value >0.5 is considered an acceptable identification according to manufacturer's instructions.
[b] T5 was later found to be a mixture of 2 species, T5A and T5B.

Table 2. Qualitative results of microbes from vermiculite inhabited by *C. formosanus* plated on nutrient agar

Plate	Comments	Original isolation	Gram reaction (+ or −)	Closest species ID	Similarity[a]
V1	Cream-colored colonies	Nutrient agar	−	*Enterobacter gergoviae*	0.626
V2	Cream-colored colonies	Nutrient agar	+	*Corynebacterium urealyticum*	0.517
V3	Cream-colored colonies	Nutrient agar	−	*Acinetobacter calcoacet/baumannii/Gen2*	0.791
V4	Cream-colored colonies	Nutrient agar	−	*Acinetobacter calcoacet/baumannii/Gen2*	0.814
V5	Red-colored colonies	Nutrient agar	−	*Serratia marscescens*	0.724
V6	Cream-colored colonies	VRBA	−	*Enterobacter* gergoviae	0.586
F_1	Fungus	Water agar	NA		

NA, not applicable.
[a] A similarity value >0.5 is considered an acceptable identification according to manufacturer's instructions.

used as a control. Plates containing termites were incubated at ≈100% RH and 24°C.

Termite mortality was evaluated three times a week for ≈3 wk, or until control mortality became excessive. Dead termites were not removed from the units. Cumulative mortality (mean % and standard error) was calculated for each treatment. Treatments from the same experiment were compared using analysis of variance (ANOVA) following transformation by the arcsine of the square of the root proportional mortality. When significant differences occurred ($\alpha < 0.05$) means were separated using Fisher least significant difference (LSD) multiple range test ($\alpha = 0.05$), (PROC GLM, SAS Institute 1990). Actual percent mortality was reported in the tables.

Lethal time to mortality was calculated by pooling data from the six replicates and analyzed by probit analysis (Finney 1971). LT_{50}s and LT_{90}s with their confidence intervals (±95% CI) were estimated for three bacterial strains in experiment 2. Two lethal treatment values were considered significantly different when their 95% fiducial limits failed to overlap. Comparisons of the slopes were made using a likelihood ratio test of parallelism where the slopes of the probit lines are constrained to the same line. The hypothesis that the lines are parallel was not rejected when $P > 0.05$ and relative potencies were calculated according to Robertson and Preisler (1992). A tolerance ratio was considered significant if the 95% CL excluded one.

Results and Discussion

Termite Microbial Isolates. All initial streaked plates showed pink colonies after 24 h of incubation on the bench top (≈23°C). Qualitative results of bacteria isolated from the *C. formosanus* cadavers indicate they were all gram-negative (Table 1). *Serratia marcescens* Bizio was the primary bacterium isolated with differences in similarity values indicative of multiple strains of *S. marcescens* (Table 1). *S. marcescens*, a nonspore forming bacterium, is generally thought to cause little harm to termites unless the insects were exposed to certain stress factors. *S. marcescens* is common in the digestive tracts of many insects but seldom causes pathogenic infection because this bacterium lacks the invasive power to penetrate through the midgut wall (Burges and Hussey 1971). Lund (1965), however, found *S. marcescens* variety *klensis* had a high degree of pathogenicity to *Reticulitermes* sp., with a single infected insect transmitting the disease to an entire laboratory colony. Strain differences of *S. marcescens* have large effects on pathogenicity (Lund 1965, O'Callaghan et al. 1996). Finding primarily one species of bacterium among the termite isolates is an indica-

Table 3. Efficacy of bacteria strains on *Coptotermes formosanus* (mean ± SE)

Strain	% mortality[a] days							
	2	5	7	9	12	14	16	19
T1	0A	3.3 ± 3.3BC	3.3 ± 3.3A	6.7 ± 6.7ABC	6.7 ± 6.7A	6.7 ± 6.7A	10.0 ± 5.8A	13.3 ± 8.8A
T2	0A	0C	0A	0C	0A	0A	3.3 ± 3.3A	3.3 ± 3.3A
T3	0A	0C	0A	0C	0A	0A	0A	0A
T4	0A	0C	0A	0C	0A	3.3 ± 3.3A	3.3 ± 3.3A	6.6 ± 6.7A
T5	0A	13.3 ± 8.8A	13.3 ± 8.8A	16.7 ± 8.8A	16.7 ± 8.8A	16.7 ± 8.8A	20.0 ± 5.8	23.3 ± 6.7A
T6	0A	0C	0A	0C	3.3 ± 3.3A	3.3 ± 3.3A	3.3 ± 3.3A	3.3 ± 3.3A
T7	0A	0C	0A	0C	0A	0A	0A	0A
T8	0A	0C	0A	0C	20.0 ± 15.3A	20.0 ± 15.2A	23.3 ± 14.5A	23.3 ± 14.5A
T9	0A	0C	0A	0C	3.3 ± 3.3A	3.3 ± 3.3A	3.3 ± 3.3A	3.3 ± 3.3A
V1	0A	6.7 ± 3.3AB	6.7 ± 3.3A	13.3 ± 6.7AB	13.3 ± 6.7A	13.3 ± 6.7A	13.3 ± 6.7A	13.3 ± 6.7A
V3	0A	0C	0A	0C	0A	0A	0A	0A
V4	0A	0C	0A	0C	3.3 ± 3.3A	16.7 ± 12.0A	16.7 ± 12.0A	16.7 ± 12.0A
V5	0A	0C	0A	3.3 ± 3.3BC	6.7 ± 6.7A	10.0 ± 8.8A	13.3 ± 8.8A	13.3 ± 8.8A
V6	0A	0C	0A	0C	0A	0A	0A	0A
UNT	0A	0C	0A	0C	0A	3.3 ± 3.3A	3.3 ± 3.3A	6.6 ± 3.3A

Within the same column means followed by the same letter are not significantly different ($P > 0.05$), LSD test. UNT, untreated control.
[a] Ten workers (more than third instar) per replicate with three replicates per treatment.

Table 4. Efficacy of *Serratia marcescens* strains on *Coptotermes formosanus* (mean ± SE)

Strain	% mortality[a] days							
	2	5	7	9	12	14	16	19
T5	13.3 ± 6.1AB	33.3 ± 16.9BC	50.0 ± 16.7A	66.7 ± 14.1AB	75.0 ± 13.6A	90.0 ± 5.2A	91.7 ± 5.4A	95.0 ± 3.4A
T8	38.3 ± 12.0A	78.3 ± 12.8A	85.0 ± 9.6A	90.0 ± 10.0A	93.3 ± 6.7A	95 ± 5.0A	95.0 ± 5.0A	100A
V5	25.0 ± 14.3AB	48.3 ± 18.9AB	50.0 ± 19.7A4	53.3 ± 19.6B	55.0 ± 20.3A	71.7 ± 18.0A	86.7 ± 11.5A	88.3 ± 11.7A
UNT	0B	1.7 ± 1.7C	5.0 ± 2.2B	5.0 ± 2.2C	8.3 ± 4.0B	10.0 ± 4.5B	10.0 ± 4.5B	11.7 ± 3.1B

Within the same column means followed by the same letter are not significantly different ($P > 0.05$), LSD test. UNT, untreated control.
[a] Ten workers (more than third instar) per replicate with six replicates per treatment.

tion of antagonism between different bacteria, thus preventing mixed infections (Krieg 1971).

Although mechanisms of pathogenicity in these bacteria have not been elucidated, *S. marcescens* produces extracellular proteases (Lysenko and Kucera 1971). This was evident in *C. formosanus* cadavers infected with *S. marcescens*, where the abdomens rapidly lost their integrity and degraded into a wet, amorphous mass. The mode of pathogenicity of these bacteria to termites requires further examination. Little is known about factors influencing pathogenicity and few investigations have been made to develop their use in microbial control of insects (Falcon 1971).

Vermiculite Microbial Isolates. Four species of bacteria and one fungus were isolated from the termite-inhabited vermiculite. Qualitatively, bacterial isolates were either cream-colored or red (Table 2). Bacteria were identified as *Enterobacter gergoviae* Brenner, *Corynebacterium urealyticum* Pitcher, *Acinetobacter calcoacet* (Beijerinck), or *S. marcescens.* The fungus was identified as *Cunninghamella echinulata* (Thaxter) (Table 2). Vermiculite controls produced no microbial growth.

Bacteria in the family Enterobacteriaceae are mutualistic gut bacteria that may invade the body cavity (Krieg 1971). These bacteria are also distributed worldwide and are found in soil, water, fruits, vegetables, grains, flowering plants, and trees. They have been isolated in animals including worms, insects, and humans (Holt et al. 1994). *E. gergoviae* is an opportunistic human pathogen, causing burn, wound, urinary tract infection, and occasionally septicemia and meningitis (Holt et al. 1994). *Corynebacterium urealyticum* was the only gram-positive bacterium isolated (Table 2). *Corynebacterium* spp. are primarily obligate parasites of mucous membranes or skin of mammals with some species being pathogenic to man, e.g., *Corynebacterium diphtheriae* (Holt et al. 1994). Multi-antibiotic resistant *Corynebacterium urealyticum* has been found responsible for urinary tract infections in man. *Acinetobacter* sp. occurs naturally in soil, water, and sewage (Holt et al. 1994). Pest control professionals, construction contractors, and termitologists working closely with these insects should note the occurrence of potential human pathogens associated with *C. formosanus.*

Health concerns for humans must be acknowledged in the development of biological control agents. *S. marcescens* is known to be a prominent opportunistic pathogen for immune compromised humans, causing septicemia and urinary tract infections (Holt et al. 1994). *S. marcescens* pathogenic to humans are generally nonpigmented strains and usually limited to hospital environments (O'Callaghan et al. 1996).

Termite Bioassays. In experiment 1, mortality was significantly higher than controls in T5 and V1 at days 5 ($F = 2.56$; df = 14, 30; $P < 0.05$: LSD; $P < 0.05$) and day 9 ($F = 2.31$; df = 14, 30; $P < 0.05$: LSD: $P < 0.05$) (Table 3). Strains T5, T8, and V5 were bioassayed in experiment 2 (Table 4). Strains T5 and T8 were selected because of numerically higher mortality. V5 was selected, because it was the only vermiculite isolated *S. marcescens* with red pigment believed not to be pathogenic to humans (O'Callaghan et al. 1996). Termites killed by T5, T8, and V5 in experiment 2 appeared red, implicating *S. marcescens* as the agent responsible for mortality. Strain T8 was significantly more active than controls at 2 d ($F = 3.24$; df = 3, 20; $P < 0.05$: LSD; $P < 0.05$) with 100% mortality by 19 d (Table 4; Fig. 1). T8 had a lethal time to 90% mortality of 10.2 d compared with 20.1 and 82.2 d for T5 and V5, respectively (Table 5). T5 and T8 had parallel log-dose probit-mortality slopes and V5 had a significantly flatter slope (Table 5). T5 killed significantly faster (2.6×) than V5 (95% CI nonoverlapping). T5 had significantly greater mortality than the control by 7 d ($F = 5.46$; df = 3, 20; $P < 0.001$: LSD; $P < 0.05$) and displayed 95.0% mortality by 19 d (Table 4; Fig. 1). After additional bacterial isolation, T5 was found to be a mixture of two bacteria, *S. marcescens* and

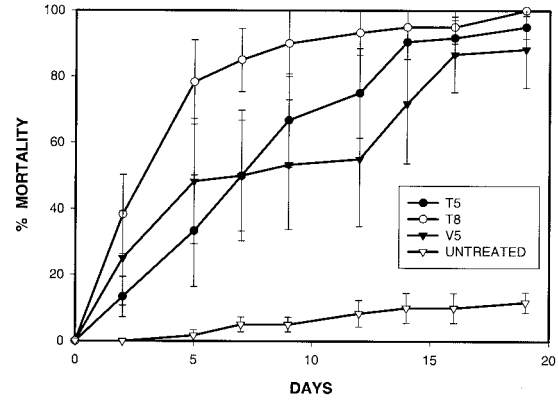

Fig. 1. Virulence of *Serratia marcescens* (1.89×10^8 CFU/cm^2) to *Coptotermes formosanus* (mean ± SE).

Table 5. Lethal time to mortality of *Coptotermes formosanus* exposed to *Serratia marcescens*

Strain	*n*	Slope ± SE[a]	LT_{50} (95%FL) (days)	LT_{90} (95%FL) (days)	χ^2	Relative potencies LT_{90} (95% CI)[b]
T5	60	2.9 ± 0.3A	7.3 (6.5–8.1)	20.1 (16.9–25.6)	1.7	1.1 (0.8–1.6)
T8	60	2.8 ± 0.3A	3.6 (3.0–4.2)	10.2 (8.8–12.1)	1.4	2.6 (1.8–4.0)
V5	60	1.2 ± 0.2B	6.9 (5.2–8.6)	82.2 (45.5–259.5)	4.8[c]	1.0

n, sample size.
[a] Slopes followed by the same letter indicate the hypothesis that the lines are parallel cannot be rejected when $P > 0.05$.
[b] Tolerance ratio and confidence interval (±95% CI) calculated by the method of Robertson and Preisler (1992).
[c] Chi-square exceeds tabular $P > 0.05$ value.

Klebsiella terrigena Izard et al. (Table 1). Why greater termite mortality occurred in experiment 2 is not understood. Further investigations of these strains are warranted.

Subterranean termites are social insects and live closely together in a moist, warm microenvironment. These conditions could support an epizootic, enhancing the potential for biological control. The culturing and identification of microbes associated with moribund *C. formosanus* supports the investigation of a biological control strategy involving stimulation of the endemic natural pathogens or opportunistic microorganisms to weaken the colony (Ferron 1978). Such a strategy must overcome the mechanisms that have evolved to protect the colony from an epizootic, such as grooming, the ability to isolate infected colony members by walling off diseased termites, and possible repellency of microbes to termites (Wood and Sands 1978, Su et al. 1982). More sophisticated bioassays and field tests are required to evaluate the behavioral response of termites to biological control agents. Our interest was to determine virulence of the bacterial isolates associated with *C. formosanus* and bioassay the most promising isolates as potential biological control agents. *S. marcescens* strains T5, T8, and T5 provided substantial mortality of *C. formosanus* in the confines of a plate test. Further investigations involving the use of bacteria synergised with chemical agents are being conducted.

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